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ALPHA-TOCOPHEROL: USES IN PREVENTING NITROSAMINE FORMATION

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INTRODUCTION

There are numerous reports in the literature concerning the *in vivo* and *in vitro* formation of nitrosamines resulting from the reaction of nitrite (HONO, N_2O_3, NO_2^-, NO^+) with susceptible amines (Bogovski, & Walker, 1974). The amine substrates studied include drugs, as well as naturally occurring compounds present in food. Approximately 80% of the nitrosamines studied have been found to be potent animal carcinogens and hence pose a particular danger to man as potential carcinogens (Lijinsky, 1977).

Several authors have reported on the ability of ascorbic acid (vitamin C) to inhibit nitrosamine formation. Mirvish et al. (1972) have suggested that the potential danger of ingesting drugs or certain food substances, which can be nitrosated, may be reduced by the addition of ascorbic acid. Kamm et al. (1973) have shown that ascorbic acid does protect against hepatotoxicity in rats caused by the simultaneous feeding of sodium nitrite and aminopyrine. Fiddler et al. (1973) demonstrated that the incorporation of ascorbate and/or erythorbate into the curing mixture used to prepare frankfurters greatly reduced the amount of nitrosodimethylamine (NDMA) in the end product.

The mechanism of the protective effect of ascorbic acid is currently believed to be due to the ability of the latter to compete with susceptible amines for the available nitrite ion (i.e. nitrosating species). This premise is based on the work of Dahn et al. (1960), who studied the anaerobic oxidation of ascorbic acid by nitrous acid (HONO). These workers reported that the initial attack by the nitrosating species is on the 3-hydroxy group of ascorbic acid, forming the nitrite ester, which subsequently decomposes to yield the semiquinone. Further reaction of the semiquinone with an additional mole of nitrosating species completes the oxidation of ascorbate to dehydroascorbic acid.

Archer et al. (1975) suggested that between pH 1.5 and 5.0, the anaerobic nitrosation of secondary amines in the presence of ascorbic acid involves two competitive second order reactions as follows:

$$\text{Amine} + N_2O_3 \xrightarrow{k_1} \text{Nitrosamine} + NO_2^- + H^+ \qquad (1)$$

$$\text{Ascorbate} + N_2O_3 \xrightarrow{k_2} \text{dehydroascorbic acid} + 2NO + 2H_2O \qquad (2)$$

If $k_2 \gg k_1$, then ascorbate will successfully compete with amine for available nitrite. The above reaction scheme also suggests that any reducing agent capable of rapidly destroying nitrite could be a potential protective agent against nitrosamine formation.

The reaction of an amine to form a nitrosamine requires that a nitrosating species be present. Three major sources of nitrite in man's environment have been identified. Nitrite is deliberately added to certain consumer products to achieve desired flavor, color and preservation; nitrite is formed in human saliva by microbiological reduction of salivary nitrate; and finally, nitrite is present in the atmosphere, as oxides of nitrogen, as a result of the combustion of organic and inorganic nitrogen compounds, e.g. from automobile exhaust, industrial smoke stack emission and cigarette smoke.

A model system has been developed in our laboratory to evaluate the ability of reducing agents to destroy nitrite ion, relative to that of ascorbic acid. The present paper deals with the studies performed to evaluate the performance of α-tocopherol, a fat-soluble vitamin.

MATERIALS AND METHODS

Water-dispersible forms of tocopherol

α-Tocopherol (vitamin E) is a viscous oil which is essentially insoluble in water. In order to facilitate dispersion of this compound in an aqueous, *in vitro*, model system and for *in vivo* experiments, a water-dispersible form was prepared. The oil was emulsified in water, using a food grade "Dextrin"-type emulsification

agent, and subsequently spray-dried to yield a free-flowing solid which contained 33% by weight of α-tocopherol.

In addition to the above preparation, a placebo was also made which contained Neobee M-5[1] (a fractionated, medium-chain triglyceride of coconut oil origin, free of tocopherols) in place of α-tocopherol, to ensure that the resuspended spray-dried product would show all the characteristics of an oil-in-water emulsion which are present in the tocopherol preparation. Similar preparations were also prepared for γ-tocopherol and α-tocopherolquinone, a known oxidation product of vitamin E. For some *in vitro* studies, α-tocopherol was dispersed in aqueous systems using Polysorbate 20 as the emulsifier. A 6:1 ratio of Polysorbate 20 to α-tocopherol forms a stable emulsion of α-tocopherol in water.

In vitro *experiments*

Model system experiments were performed in either Britton-Robinson buffer solution or USP simulated gastric fluid. Solutions were equilibrated at 37 ± 1°C in a water bath and the reaction was initiated by the introduction of sodium nitrite. Reactions were stopped by transferring 1-5 ml samples of the reaction mixture into 2 ml of 1 N sodium hydroxide and extracting NDMA into 10.0 ml of methylene chloride. Gas chromatographic analysis was performed on the methylene chloride extracts. Nitrite determinations were performed by removing a 5 µl sample from the reaction mixture and introducing it directly into 10.0 ml of nitrite reagent (Saltzman, 1954). Nitrite values were corrected for endogenous losses by including control samples (i.e. no reducing agent) in all *in vitro* experiments. Tocopherol determinations were performed by the Emmerie-Engel procedure, after clean-up by extraction of tocopherol into petroleum ether (Bunnell, 1967).

In vivo *experiments*

The animal experiments were the same as those described by Kamm (1974), except that spray dried tocopherols were substituted for sodium ascorbate. Control animals were given the vehicle without tocopherol.

Cigarette experiments

Cigarettes were prepared using 2R1 Kentucky reference tobacco. An alcoholic solution of α-tocopherol was applied to the shredded tobacco and allowed to dry. Control tobacco was treated with plain alcohol. Cigarettes were prepared using a Laredo® cigarette machine at a fill weight of 1.1 g of tobacco/85 mm cigarette and conditioned 24 hours at 60% R.H., prior to smoking on a Borgwaldt smoking machine. The tars were collected in an Elmenhorst cold trap at -80%. NDMA

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[1] Neobee M-5 is available from PVO International, Inc., Chemical Specialities Division, Boonton, NJ, 07005.

was isolated from the combined tars of 60 cigarettes by steam distillation from 2 N sodium hydroxide, extraction of the distillate into methylene chloride, followed by column chromatography on a two-stage, silica-gel (10 g), basic alumina (10 g) column. The methylene chloride was reduced in volume to 0.5 ml prior to gas chromatography. NDMA was determined by gas chromatography, using a Tracor Model 220 instrument equiped with a Tracor Model 310 (Hall) electrolytic conductivity detector. The Hall detector was operated at 400°C without a catalyst, to provide greater nitrosamine specificity. The column was a 6' x 1/4" 10% Carbowax 20M glass column, operated isothermally at 135°C with a flow rate of 50 cc/min of helium. Under these conditions, the retention time of NDMA was approximately 7 minutes.

Bacon studies

Bacon samples were fried and analysed for nitrosamines by procedures previously described (Doerr & Fiddler, 1977; Pensabene et al., 1974)[1]. Residual nitrite values were obtained using the AOAC procedure (Fiddler, 1977). Ascorbic acid determinations were performed using the Deutsch procedure (Newmark et al., 1974). For the tocopherol determinations, the homogenized samples of lean and fat portions from the bacon bellies were saponified and extracted by the method of Bieri et al. (1961). The method was modified by use of 1.0 g of 4-hydroxyacetanilide in the saponification instead of pyrogallol. Samples (7 to 10 g) were dispersed in 75 ml of ethanol and 1 g of potassium hydroxide per gram of fat was added for saponification. The extraction procedure used 75, 75 and 50 ml of hexanes (Skellysolve B). Florex chromatography and colorimetry were as described in the Analytical Methods Committee Report (1959).

RESULTS

Several experiments were performed to evaluate the ability of tocopherol to react with nitrosating intermediates generated in different phases. 2.9×10^{-4} moles of sodium nitrite was added to 10 ml aliquots of 0.1 N hydrochloric acid and immediately blanketed with 10.0 ml of Neobee m-5 oil containing known amounts of tocopherol. Neobee M-5 is a saturated coconut oil previously demonstrated to be unreactive toward nitrosating agents. The samples were incubated for one hour at 37°C without agitation and subsequently analysed for remaining tocopherol and nitrite. The results are recorded in Table 1 and indicate that tocopherol can react with the nitrosating intermediate in an aprotic solvent. Table 1 also indicates that 1 mole of tocopherol reacts with 2 moles of nitrite under these conditions.

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[1] The bacon processing and the subsequent analysis of fried bacon for volatile nitrosamines was carried out at the Eastern Regional Research Center.

Table 1. Reaction of α-tocopherol (TOC) with NO_2^- in a lipophilic medium at 37°C

Expt	Initial concentration of [TOC] in Neobee oil (M)	Initial concentration of [NO_2^-] in 0.1N HCl (M)	Δ[TOC][a] (M)	Δ[NO_2^-][a] (M)
A	5.14×10^{-2}	2.89×10^{-2}	0.66×10^{-2}	1.48×10^{-2}
B	2.58×10^{-2}	2.89×10^{-2}	0.70×10^{-2}	1.35×10^{-2}

[a] Δ - Change in reactant concentration after 1 hr

The reactions of nitrite ion with α-tocopherol in aqueous solution were studied using a weight of sample equivalent to 1.6×10^{-4} moles of tocopherol dispersed in 20 ml of simulated gastric fluid or Britton-Robinson Buffer equilibrated at 37° ± 1°C. The reaction was initiated by the addition of 1.45×10^{-4} moles of sodium nitrite. Aliquots of the reaction mixture were withdrawn at predetermined time intervals for analysis of nitrite ion and tocopherol.

Figure 1 shows the effect of the spray-dried α-tocopherol powder on nitrite ion as a function of pH. At pH 2 or 3, the reaction proceeds quite rapidly, as indicated by a marked decrease in nitrite ion concentration with time. As the pH is increased, the reaction of α-tocopherol and nitrite slows down until, at pH 5, less than 5% of the initial nitrite has disappeared after sixty minutes of reaction time at 37°C. Similar results were obtained when tocopherol was dispersed in aqueous solution with Polysorbate 20.

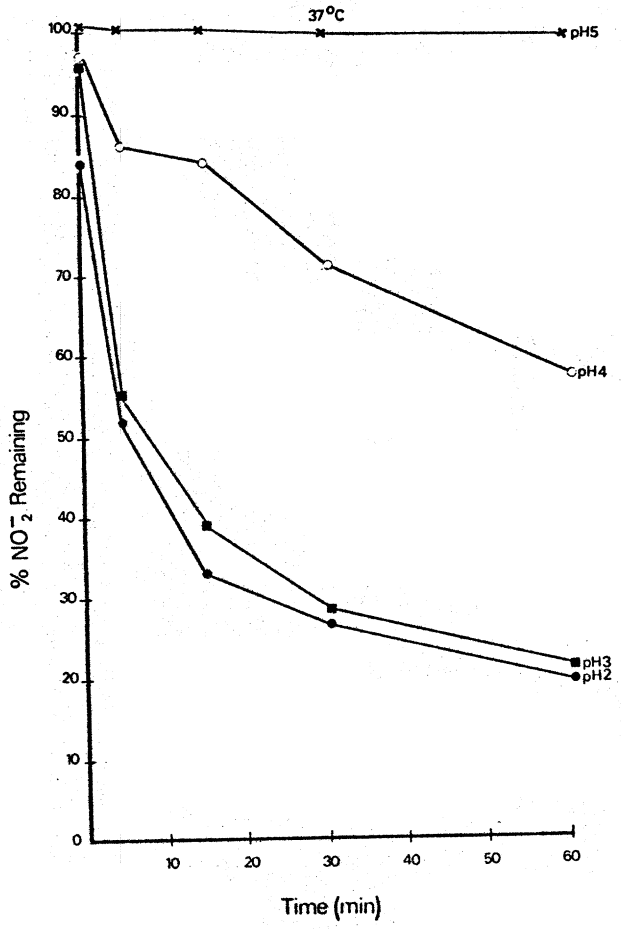
Table 2 contrasts the abilities of ascorbic acid and α-tocopherol to destroy nitrite ion. Solutions were sampled after 30 minutes at 37°C. The data indicate that α-tocopherol reacts more efficiently with nitrite than does ascorbic acid when the pH falls below 4.

Figure 2 shows the results of additional experiments in simulated gastric fluid in which α-tocopherol is compared with two closely related compounds: γ-tocopherol and α-tocopherolquinone, a known oxidation product of α-tocopherol. The results demonstrate that the water-dispersible forms of both α and γ-tocopherol are effective nitrite scavengers. α-tocopherolquinone, on the other hand, is completely unreactive toward nitrite. A similar experiment, not recorded in figure 2, showed that the Neobee oil placebo was also unreactive toward nitrite ion.

The destruction of nitrite by α-tocopherol in simulated gastric fluid suggested that α-tocopherol might also prevent the formation of

FIG. 1. EFFECT OF α-TOCOPHEROL (8.0 mM) ON NITRITE (7.0 mM) IN AQUEOUS SUSPENSIONS AT VARIOUS pH

Each point is the mean for duplicate determinations. The results are typical of two separate experiments.



nitrosamines by the action of nitrite on aminopyrine in the rat stomach, as demonstrated for ascorbic acid by Kamm et al. (1973). The results of these experiments (Table 3) demonstrate that the elevation of serum glutamic pyruvic transaminase (SGPT) normally observed in animals treated with aminopyrine plus sodium nitrite was completely prevented when α-tocopherol was included in the treatment. γ-Tocopherol, when employed at the same molar concentration as α-tocopherol, protected only 75% of the animals. The administration of

Table 2. Effect of α-tocopherol and ascorbate on nitrite as a function of pH

pH	% NO_2^- remaining after 30 minutes at 37°C	
	Ascorbate	α-Tocopherol
2	49	26
3	40	27
4	64	70
5	73	96

Initial concentrations: ascorbate = α-tocopherol = 8.0 mM
sodium nitrite = 7.25 mM

FIG. 2. EFFECT OF SPRAY-DRIED, WATER-DISPERSIBLE FORMULATIONS OF α-TOCOPHEROL (8.0 mM), γ-TOCOPHEROL (8.0 mM) AND α-TOCOPHEROLQUINONE (8.0 mM), ON NITRITE (7.0 mM) IN SIMULATED GASTRIC FLUID (pH 1.3)

Each point is the mean for duplicate determinations. The results are typical of two separate experiments.

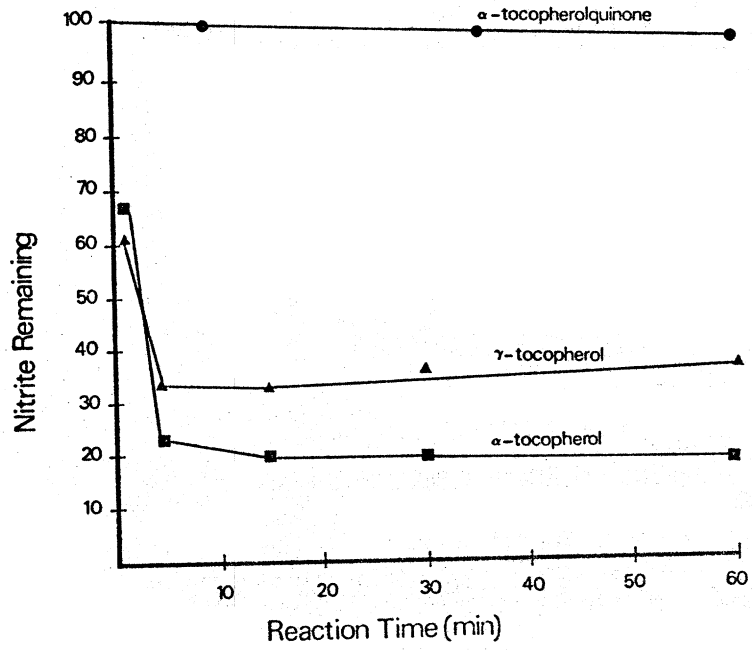


Table 3. Effect of α-tocopherol, γ-tocopherol and α-tocopherolquinone on elevation of SGPT activity in rats administered sodium nitrite plus aminopyrine[a]

Compound administered (mmol/animal)						
Sodium nitrite	Aminopyrine	α-Tocopherol	γ-Tocopherol	α-Tocopherolquinone	SGPT	
0	0	0[b]	0[b]	0[b]	25 ± 1	[0/30]
0.43	0.15	0[b]	0[b]	0[b]	216 ± 23[c]	[29/29]
0.43	0.15	0.51	-	-	20 ± 1[d]	[0/9]
0.43	0.15	-	0.51	-	47 ± 16[d]	[5/20]
0.43	0.15	-	-	0.51	207 ± 33[c]	[10/10]

[a] SGPT values (international units/liter of serum) are means ± S.E. for all animals in the group. Figures in brackets are the number of animals with an elevated SGPT relative to the total number of animals in the group. An SGPT value was considered elevated if it exceeded the mean for the controls + 2 S.E.

[b] These animals were administered placebo suspension equivalent by weight to the amount of suspension given to α-, γ-tocopherol or α-tocopherolquinone treated animals.

[c] $p < .001$ compared to animals treated with placebo only

[d] Not significantly different from animals treated with placebo only

α-tocopherolquinone was found to be totally ineffective. A more detailed presentation of these animal experiments is in press (Kamm et al., 1977).

Tobacco smoke is an aerosol composed of a physical mixture of gases and vapors in which billions of particles are dispersed. Condensed cigarette smoke (tar) has been reported to contain a variety of nitrosamines (McCormick & Underwood, 1973) including NDMA. NDMA is presumably formed by the action of nitrogen oxides (as N_2O_3) on dimethylamine. A series of experiments were conducted to determine whether α-tocopherol would inhibit NDMA formation in this system. Treated cigarettes containing 30 mg of α-tocopherol were compared with similarly prepared control (untreated) cigarettes, the results of these studies (Table 4) indicate an average reduction of 50% in NDMA yields from α-tocopherol treated cigarettes compared with controls.

Fried bacon is another system known to contain nitrosamines (Crosby et al., 1972; Fazio et al., 1973; Sen et al., 1973). The presence of nitrosamine precursor in adipose, but not lean, tissue (Fiddler et al., 1974), suggests that a nitrite scavenger in the lipophilic phase might be useful in preventing nitrosamine formation. In the study reported here, two matched pairs of fresh pork bellies were divided into thirds, forming twelve sections. α-Tocopherol was added to the standard cure solution in a ratio of 5:2 with Polysorbate 20 emulsifier, prior to pumping. This ratio was found to deliver satisfactory levels of α-tocopherol to the fat portion of the

Table 4. Effect of α-tocopherol on the NDMA yield of cigarettes

Cigarette type	NDMA (ng/cig.)		Number of determinations[a]
	Avg.	Range	
Treated[b]	7.9	(6.5-10.0)	6
Untreated	15.2	(12.7-19.7)	5

[a] Each determination represents the tars from 60 cigarettes

[b] 30 mg α-tocopherol/cigarette

belly. The theoretical sodium nitrite, ascorbate and α-tocopherol inputs for each section are recorded in Table 5, along with the overall distribution of each component in the belly. These analyses were

Table 5. Distribution of nitrite, sodium ascorbate and α-tocopherol in bacon prior to frying

Belly pair	Section	Theoret-input[a] ppm		Adipose ppm			Lean ppm			Overall ppm		
		AH_2	α-TOC	$NaNO_2$	AH_2	α-TOC	$NaNO_2$	AH_2	α-TOC	$NaNO_2$	AH_2	α-TOC
1	Brisket	0	0	37	ND	4	87	ND[b]	3	56	-	4
1A	Brisket	0	500	28	ND	192	86	ND	350	57	-	272
1	Center	500	0	24	75	3	84	171	2	47	149	2
1A	Center	500	500	23	48	175	76	255	331	53	135	242
1	Flank	0	0	31	ND	3	115	ND	4	61	-	3
1A	Flank	500	0	29	98	5	72	250	3	47	162	4
2	Brisket	0	500	33	ND	316	67	ND	319	46	-	313
2A	Brisket	500	500	10	82	229	42	205	399	24	136	304
2	Center	0	0	25	ND	3	64	ND	4	42	-	3
2A	Center	0	500	29	ND	197	88	ND	342	53	-	256
2	Flank	500	0	20	77	4	86	239	7	44	131	5
2A	Flank	500	500	13	59	220	46	174	266	23	93	232

[a] AH_2 = Sodium ascorbate; α-TOC = α-Tocopherol; $NaNO_2$ input = 125 ppm in all samples

[b] ND = Not done

performed on the same day as frying. Total (edible plus drippings) nitrosamine analyses were performed after frying and some representative results are presented in Table 6. When comparing different treatments, samples for frying were taken from the same sections of each belly side. α-Tocopherol was found to significantly inhibit nitrosopyrrolidine (NPYR) formation ($P < 0.05$). Inhibition was greater with α-tocopherol used in combination with sodium ascorbate than with sodium ascorbate alone. In addition, there was evidence of a weaker inhibition of NDMA formation in the edible fried bacon.

Table 6. *N*-Nitrosamine content of fried bacon

Belly pair	Section	Theoret-input (ppm)[a]		Nitrosamines (ppb)[b,c] Edible		Dripping	
		AH_2	TOC	NDMA	NPYR	NDMA	NPYR
1	Brisket	0	0	4	16	7	19
1A	Brisket	0	500	6	4	7	7
1	Center	500	0	7	16	17	22
1A	Center	500	500	3	6	10	10
1	Flank	0	0	5	16	10	15
1A	Flank	500	0	3	8	10	18
2	Brisket	0	500	5	4	8	8
2A	Brisket	500	500	2	4	9	5
2	Center	0	0	5	13	13	25
2A	Center	0	500	3	6	10	14
2	Flank	500	0	3	8	16	17
2A	Flank	500	500	3	4	10	7

[a] $[NaNO_2]$ = 125 ppm

[b] Corrected for recovery of nitrosoethylmethylamine internal standard

[c] Confirmed by GLC/high-resolution MS.

DISCUSSION

The *in vitro* experiments presented here make use of the inherent instability of nitrite ion in acid solution to generate the nitrosating intermediate. This is described by the following equilibrium expressions:

$$NO_2^- + H^+ \rightleftharpoons HONO \qquad (3)$$

$$2HONO \rightleftharpoons N_2O_3 + H_2O \qquad (4)$$

The mechanism whereby tocopherol is capable of reacting with a nitrosating intermediate is not fully understood. The results of our experiments indicate that tocopherol can react with nitrite when emulsified in an acidified aqueous solution and, indeed, even when dissolved in a lipophilic medium exposed to N_2O_3 which is generated in an aqueous phase. This raises an interesting question concerning the nature of the tocopherol-nitrite interaction when the former is emulsified in an aqueous system, using either Polysorbate 20 or the modified corn-starch dextrin emulsifier used in the spray-dried tocopherol preparation. Is the nitrosating species NO^+, with attack on the hydroxy group of the tocopherol molecules on the surface of the lipid micelle, or is the reactant gaseous N_2O_3 which is capable of migrating into the lipid phase? The data obtained in this study suggest that the reaction could take place totally within the micelle, as demonstrated by the ability of tocopherol to react with the nitrosating agent in Neobee oil. We also have observed that one of the reaction products of α-tocopherol is α-tocopherolquinone. This product has been qualitatively identified in the reaction of tocopherol with nitrite under both lipophilic and aqueous conditions.

The animal studies which we have reported demonstrate that only the reduced form of the tocopherols are capable of preventing amine nitrite-induced hepatotoxicity. In this respect, the mechanism of the protective effect of both α and γ-tocopherol is analogous to that previously reported for ascorbic acid (Kamm et al., 1973). This same conclusion is suggested by our *in vitro* studies in simulated gastric fluid (Fig. 2), which show destruction of nitrite by the tocopherols but not by α-tocopherolquinone. It is interesting to note that the reduced *in vitro* reactivity of γ-tocopherol toward nitrite, compared to α-tocopherol, appears to be reflected in the animal study as well in the prevention of nitrosamine formation *in vivo*.

The effectiveness of tocopherol as a nitrite scavenger has an important implication. The oxides of nitrogen, principally nitrogen dioxide, constitute not only an atmospheric source of nitrosating agent but nitrogen dioxide itself is also a lung toxicant. Studies in our laboratory have demonstrated that tissue levels of tocopherol, unlike ascorbic acid, can be raised over a wide range in many organs of a rat by feeding supplemental α-tocopherol acetate in the diet. The tissue levels of α-tocopherol increase linearly as the log of the dose for such important target organs as the lung and stomach.[1] Protection of the lung against exposure to the toxic effects of nitrogen dioxide has already received some attention (Menzel et al., 1972), similarly, it might be worthwhile to study the ability of increased stomach levels of tocopherol to protect against nitrosamine formation.

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[1] L. Machlin (1976), Hoffmann-La Roche Inc., personal communication

The addition of α-tocopherol to bacon or cigarettes represents an application to systems which have some lipophilic character; namely, the fat phase of bacon and the smoke aerosol. Although these studies are not complete, we feel the consistent reduction of NDMA in cigarette tars and of NPYR in fried bacon demonstrate the potential utility of α-tocopherol in preventing nitrosamine formation. A recent study (Walters et al., 1976) on bacon cured with α-tocopherol, in combination with ascorbyl palmitate and citric acid, supports the present findings that α-tocopherol will inhibit NPYR formation in fried bacon.

SUMMARY

α-Tocopherol has been evaluated as a nitrite scavenger for the prevention of nitrosamine formation in a model system and under practical conditions. α-Tocopherol was found to react with nitrosating agents in both lipophilic and aqueous environments. The use of α-tocopherol was shown to inhibit aminopyrene-nitrite induced hepatotoxicity in rats and to reduce the amount of NDMA formed in cigarette smoke. Of primary interest is the finding of a significant reduction of NPYR formation in fried bacon. Nitrosamine inhibition was greater with α-tocopherol used in combination with sodium ascorbate than with sodium ascorbate alone.

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NOTE: Precaution should be exercised in the handling of nitrosamines since they are potential carcinogens.

Reference to brand name or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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